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INTRODUCTION

Prostate cancer is a difficult disease to treat due to its molecular heterogeneity and diverse clinical outcomes. Current therapies for treating and diagnosing prostate cancer are unsatisfactory, suggesting that new strategies and molecular markers are greatly needed. Tumor cells express specific cell surface receptor complexes for rapid growth and survival. Specific receptor-ligand complexes have profound biological functions such as cell signaling and growth. For example, androgen receptor complex plays a critical role in prostate tumor growth and response to hormone therapy. It is important that more such complexes are identified for this disease. We propose to identify specific receptor-ligand pairs for prostate cancer. We have developed a sophisticated targeting system to probe the tumor vasculature in vivo by phage display technology. We plan to inject phage peptides libraries into prostate tumor-bearing mice to identify specific peptides targeting to the tumor and not to the normal tissues. The tumor-specific peptides will be recovered and analyzed by molecular and biochemical methods. The tumor-specific peptides will be used as a bait to identify and clone the binding receptors by affinity chromatography and biochemical cell fractionation approaches. If we are successful, we will identify new biologically relevant receptor-ligand pairs that may be developed into diagnostic and/or therapeutic applications for prostate cancer.

BODY

Background:

Prostate cancer is the second leading cause of cancer death in men and it is estimated that one in six men will develop this disease during their lifetime (1). The cause of the disease is largely unknown. This is also compounded by the fact the disease is heterogeneous with diverse clinical outcomes. Studies have shown that tumors are heterogeneous comprising sub-population of tumor cells with different molecular properties and genetic alterations (2-4). Some of these different properties include growth rate, metastasis, resistance to cytotoxic drugs, and cell surface receptors (3). Moreover, the tumor microenvironment is extremely complex consisting of many cell types that can crosstalk with each other by activating and inactivating cell surface receptors (5). Tumor cells express specific cell surface receptors that can interact with growth factors and cytokines for rapid growth, survival, and cell signaling to the extracellular environment. Specific receptor-ligand complexes can have profound biological functions. For example, in the case for prostate cancer, it is well known that androgen-androgen receptor complex plays a critical role in prostate tumor growth and response to hormone therapy (6, 7). The complex specifically activates transcription of androgen-regulated genes and promotes cellular proliferation, survival, and differentiation (8, 9). However, there are a limited number of receptor-ligand pairs that have been thus far identified for prostate cancer. We propose to identify specific receptor-ligand pairs for prostate cancer by in vivo phage display. This approach has not been explored for targeting prostate tumor in vivo. Identifying the molecular receptor-ligand complexes during tumor development is an important step towards developing new diagnostic markers and molecular therapeutic targets for prostate cancer.

Statement of work:

Task 1. To isolate and characterize peptides targeting prostate tumor cells in vivo by phage peptide libraries (1-18 months).

- We will generate 30 tumor-bearing mice (human prostate cancer xenografts) for the in vivo screening (1-2 months). **Completed.**
- We will inject phage peptide libraries into the tumor-bearing mice and isolate tumor-specific homing phage peptides after three or four rounds of in vivo selection. We will use several different peptide libraries (3-7 months). **Completed.**
- We will characterize the binding and inhibition properties of the tumor homing peptides in vitro and in vivo. We will generate 30 tumor-bearing for the in vivo studies. Prostate cancer cell lines including DU145, PC-3, and LNCaP will be used for the in vitro studies (8-12 months). **Completed.**
- We will characterize the tissue localization of the tumor-specific homing phage peptides by immunohistochemistry. Tissue samples from the tumor bearing mice and prostate cell lines will be used for these studies (13-18 months). **Completed.**

Task 2. To identify and validate molecular receptors binding to the tumor homing peptides (18-36 months).

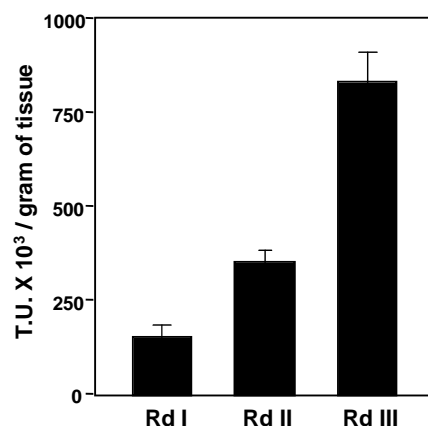
- We will analyze tumor-specific peptides by searching the protein database for potential biologically relevant receptor leads (18-20 months). **Completed.**
- We will use biochemical cell fractionation and affinity chromatography to purify the receptor binding to the tumor-specific peptides. We will select one or two most promising peptides for receptor identification (21-30 months). **Completed.**
- We will clone the receptors and characterize them in the context of the tumor homing peptides (31-36 months). **On-going.**

Accomplishments for Task 1:

We applied in vivo phage display strategy (10, 11) to identify receptor-ligand pairs in human-derived prostate cancer tumor-bearing mice. We wanted to target tumor-specific receptors that are over-expressed and/or activated during tumor development. Since our in vivo phage targeting system has been successful in targeting the blood vessels of both normal and tumor vasculature (10-21), we applied this approach with some modification to preferentially target the tumor cells and not the blood vessels. A typically tumor vasculature experiments involves the injection of the phage library into the tumor-bearing mice for a short time between five to ten minutes. Since tumor blood vessels are leaky and our goal was to specifically target tumor cells, we allowed the phage to circulate for twenty-four hours to increase the chance of targeting tumor cells and not blood vessels. We generated tumor-bearing mice using the human prostate cancer cell line DU145. We injected a phage peptide library into tumor-bearing mice intravenously and allowed the phage to circulate for twenty-four hours. The bound phage was recovered and amplified for another round of selection. We performed three rounds of in vivo selection to increase the enrichment of phage peptides specifically targeting the tumor cells. We found a significant enrichment after the third round of selection from the tumor (Figure 1).

Individual phage clones were obtained from the third of selection and the phage DNA insert was sequenced. As shown in Table 1 many tumor homing peptides were identified but only two peptides were highly enriched (fold face): YRCTLNSPFFWEDMTHECHA sequence repeated eleven times and LGCMASMLREFEGATHACTQ sequence repeated eight times. We investigated the phage peptide YRCTLNSPFFWEDMTHECHA for further analysis since this one was the most enriched peptide sequence. Interestingly, the recovered tumor homing peptides did not match to any sequences that were identified for the normal prostate organ (16).

Figure 1. In vivo screening of phage peptide library in prostate tumor-bearing mice. A $X_2CX_{14}CX_2$ (C, cysteine; X, any amino acids) peptide library (10^9 transducing units, T.U.) was injected into the tail vein and allowed to circulate for 24 hours followed by perfusion through the heart and the bound phage was recovered from the tumor. The phage recovered from the tumor was amplified and re-injected in two consecutive rounds of selection. The number of T.U. is shown for the tumor and normalized by tissue weight. The error bar shows standard deviation of the mean from triplicate plating.



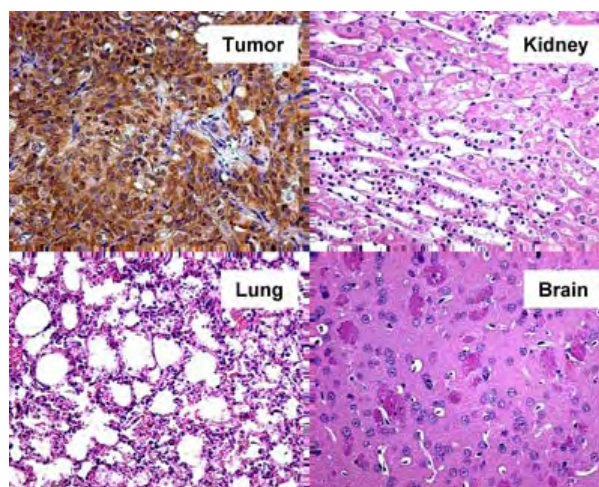
The phage peptide, YRCTLNSPFFWEDMTHECHA, was tested for its homing specificity in the human-derived DU145 xenografts. We injected the phage tumor homing peptide intravenously into the tumor-bearing mice and allowed the phage to circulate for twenty-four hours prior to harvesting the tumor and the control organs including kidney, brain, and lung. The tissues were processed for immunohistochemistry followed by immunostaining with antibody against the phage. We found a significant positive brown staining in the tumor and little to no staining in the control organs (Figure 2). This result suggested that the phage peptide, YRCTLNSPFFWEDMTHECHA, is tumor specific and does not target normal tissues in vivo.

Table 1. Peptide sequences recovered from phage that target the prostate tumor in vivo

| | | | |
|-----------------------|------|-----------------------|-----|
| YRCTLNSPFFWEDMTHECHA | (11) | NDCSAHAQPGWDEVPPMCNQ | (1) |
| LGCMAFMLREFEGATHACTQ | (8) | NNCPVEGSQQNYSGATWCRA | (1) |
| RGCTEAAGLVIGITTHQCGN | (3) | TTCNKSMSSQPMRDSRECHR | (1) |
| IGCNHPSPLGSTVVPTYCFK | (3) | TSCVRTGHDENLLKAAAYCSS | (1) |
| GTCPRQFFHMQEFWPSDCSR | (3) | TECRGASSGSVSGAATDCRD | (1) |
| DRCVLVRPEFGRGDARLCHS | (2) | TLCPPASMG LGREKPRLCVS | (1) |
| EGCSDIMNTAAERTVGDSCY | (2) | TLCRSLEHEVGLFKPRECPF | (1) |
| VFCGSGSYCGVEMLASRCGH | (2) | LRCPLEVDRPNRDP AFLCSQ | (1) |
| RECGRTVHRYPWGSPESCER | (2) | LGCNKGRYWLSTRLSVSCAL | (1) |
| DACSRFLGERVDATAAGCSR | (2) | VACDISAVERLPASARSCKT | (1) |
| GNCMGLQVSELFMGYPYKCRQ | (2) | VVCFMERQMGTDVVSPMCVN | (1) |
| SRCHALRSQSVSTAGACIS | (1) | VECVMASASTDGTAAHPCKP | (1) |
| YSCTRLNGTGLQNPPSACDR | (1) | VRCEAQLQDSGTVPHPCLR | (1) |
| WVCTSASQDTRLKEPGMCIA | (1) | PNCDDLDDIVLNPYTAGPCGT | (1) |
| MHCTSQTLRGTPSLAPKCS | (1) | PNCYSGDGEISSHIPVQCLM | (1) |
| QHCVKGQFPFRESVTITCNS | (1) | PGCVVSPFALSAQGTSTVCTI | (1) |
| HTCWGARDVAQPSGTVRCLK | (1) | GDCETNNVTKVGGITRNCVG | (1) |
| ARCREDTGFMGLGSANICTD | (1) | GYCLTVVGGAVLTIALLCVT | (1) |
| RTCEEVRNRALEELTNFCPY | (1) | GPCAATGVNPGDHGAAVCDQ | (1) |
| RTCQVRSNNISPRMALACVT | (1) | GDCETNNVTKVGGITRNCVG | (1) |
| RSCVNSDTGVLQRGAPSLCF | (1) | KSCGKYGLIVGQPPAEHCPP | (1) |
| RGCW RDSTAWHVSYPVECLA | (1) | KLCYRSSAGSEL RPPEKCA | (1) |
| NRCMPGFLDDADSAASPCGS | (1) | KICPVTNMWTTSPWAHKCGM | (1) |
| NQCSSLLTYQGWRKTKDCIP | (1) | | |

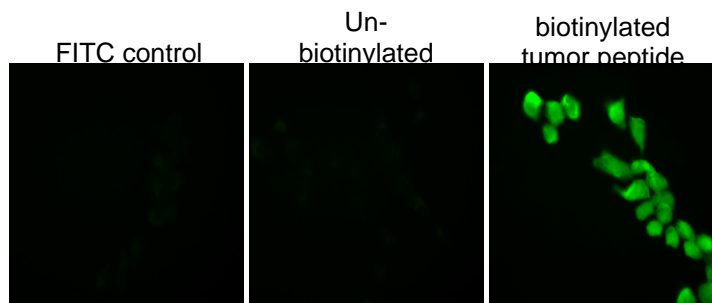
The number in the parenthesis indicates the number of times the sequence was repeated

Figure 2. Immunohistochemical staining of the tumor homing phage peptide to the prostate tumor. The phage peptide, YRCTLNSPFFWEDMTHECHA, was injected into human-derived DU145 tumor-bearing mice and allowed to circulate for 24 hours. After perfusion, the tumor and control tissues were recovered and processed for immunoperoxidase staining to detect the phage. The monoclonal anti-M13 antibody was used to stain for phage. Strong positive brown staining was detected in the tumor and negative in the control organs including the kidney, lung, and brain. The tissue sections were counter-stained with hematoxylin.



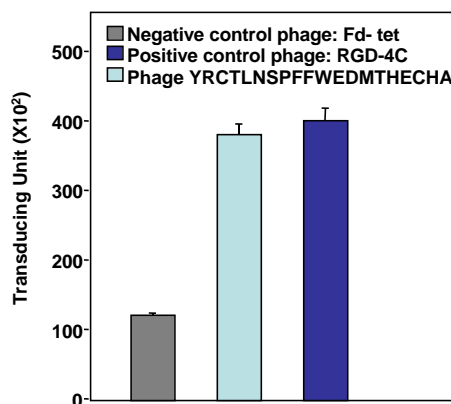
Next, we tested the localization of the tumor-homing peptide on DU145 cells *in vitro*. The cells were fixed and incubated with the tumor homing peptide that was biotinylated or un-biotinylated followed by labeling with streptavidine-FITC conjugated secondary antibody. We detected strong immunofluorescence staining with the tumor homing biotinylated peptide-YRCTLNSPFFWEDMTHECHA (Figure 3). The staining revealed both cytoplasmic and cell surface localization. We tested other tumor cell lines such as PC-3, LNCaP, and normal prostate epithelial cells. Similar results were observed.

Figure 3. Localization of tumor homing peptide on DU145 prostate cancer cells. DU145 cells were fixed with 2% paraformaldehyde on ice and incubated with the biotinylated or un-biotinylated tumor homing peptide-YRCTLNSPFFWEDMTHECHA followed by immunofluorescence detection with anti-streptavidine-FITC conjugated secondary antibody.



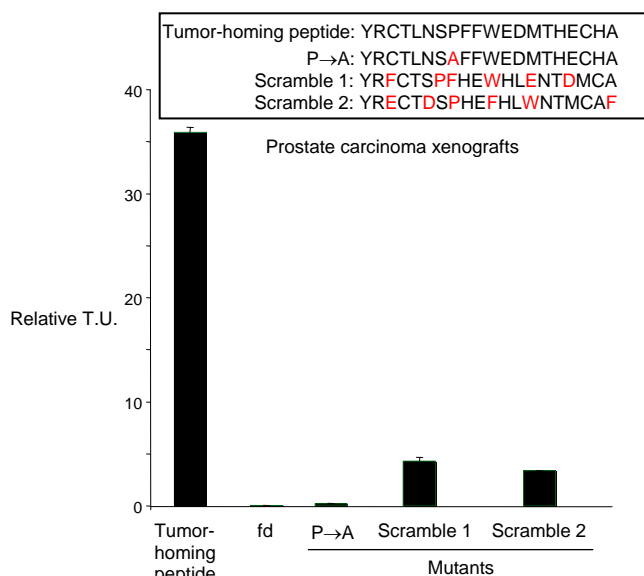
We next performed an experiment to further demonstrate the peptide binding capability on DU145 cells using the BRAZIL (Biopanning and Rapid Analysis of Selected Interactive Ligands) method (22). This is a method developed by our laboratory and it is based on an organic phase separation where phage peptides in complex with cells are separated from unbound phage by differential centrifugation through a non-miscible organic phase. In applying this method we found that the peptide YRCTLNSPFFWEDMTHECHA preferentially localized to the cell surface on DU-145 cells, whereas in the Fd-tet (insert-less phage expressing no peptide) limited binding was observed (Figure 4). We found significant binding of the phage peptide RGD-4C (positive control).

Figure 4. Cell surface binding of the tumor homing phage peptide. A suspension of DU-145 cells was incubated with the tumor homing phage peptide YRCTLNSPFFWEDMTHECHA or with control phage with no peptide displayed (Fd-tet). The phage expressing the RGD-4C was used as a positive control. The phage bound to the cells was recovered by differential centrifugation and the unbound phage remained in the upper aqueous phase. The recovered phage was counted as transducing units.



To functionally characterize the tumor-homing peptide, we made several mutants of the tumor-homing peptide and tested its homing capability *in vivo*. We made random scrambled peptides and single residue substitution constructs. We observed marked and specific tumor homing after systemic administration in prostate cancer xenografts of the tumor-homing peptide phage; in contrast, mutant constructs showed no localization to tumors (Figure 5). It was interesting to find that the single amino acid substitution of proline to alanine inhibited the tumor homing capability.

Figure 5. In vivo targeting of the tumor-homing phage, scrambled and mutant phage constructs in DU145 prostate cancer xenografts. Tumor-homing phage, YRCTLNSPFFWEDMTHECHA, or controls were administered to prostate cancer tumor-bearing mice. Only the tumor-homing phage targeted the tumor, whereas the scrambles and the mutant phage did not. Representative data from two independent experiments are shown. The red color indicates the changed residues. T.U., transducing unit of phage.



Since the tumor homing phage peptide YRCTLNSPFFWEDMTHECHA targets DU145 prostate cancer cells both in vitro and in vivo, we further investigated the targeting and internalization capability of the peptide conjugated to a known pro-apoptotic peptide KLAKKLAK. This pro-apoptotic peptide has been extensively characterized both in vitro and in vivo for its efficacy and cytotoxicity (23-25). The pro-apoptotic peptide specifically targets the eukaryotic mitochondria membranes upon internalization within cells (23).

The tumor homing peptide, YRCTLNSPFFWEDMTHECHA, was conjugated to $D(KLAKKLAK)_2$ and added with increasing concentrations to DU145 cells. Cell proliferation was measured using a modified version of the MTT assay (WST-1). We found a significant cell killing at 30 μ M with the conjugated tumor homing peptide (Figure 6), suggesting that the peptide was internalized and reached its target. We further showed that the cell death was mediated by apoptosis as shown by annexin V staining (Figure 7). A significant number of DU145 cells became apoptotic when the conjugated tumor homing peptide was added to the cells, whereas in the controls no significant apoptosis was observed. These studies suggest that the tumor homing peptide, YRCTLNSPFFWEDMTHECHA, specifically targets DU145 prostate cancer cells and has the capability to internalize in the cell.

Figure 6. In vitro targeting and internalization of the tumor homing peptide. DU-145 cells were incubated with increasing concentration of the conjugated tumor homing peptide, YRCTLNSPFFWEDMTHECHAG $D(KLAKKLAK)_2$ or control peptide, $D(KLAKKLAK)_2$. Cell viability was measured by a modified version of MTT assay (WST-1 assay).

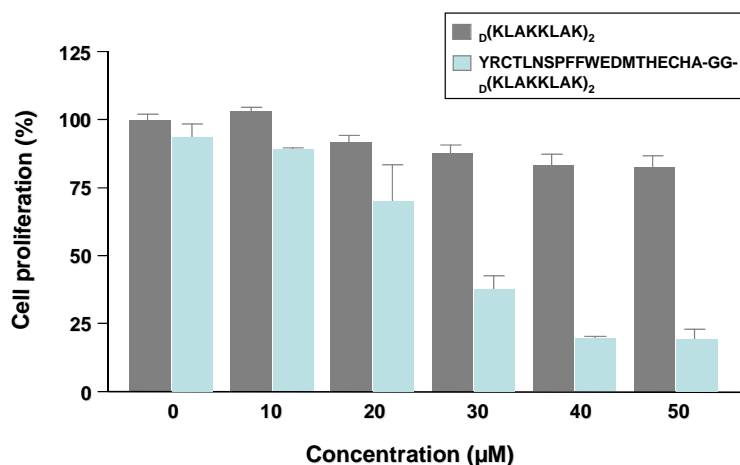
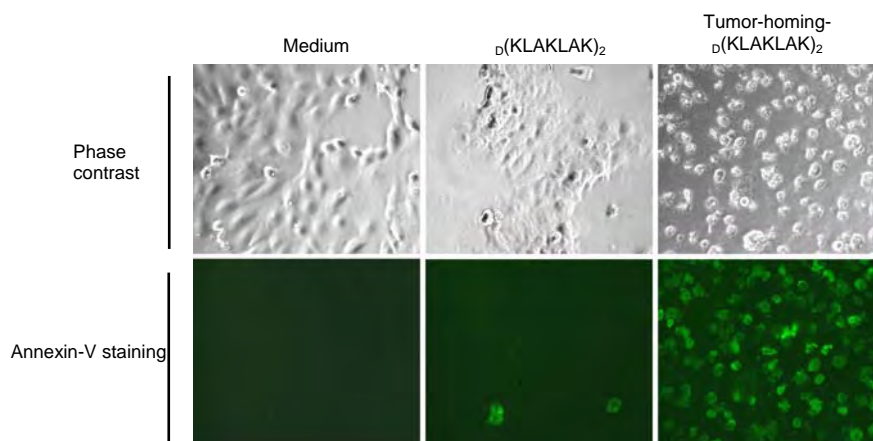


Figure 7. Induction of apoptosis by the conjugated tumor homing peptide. DU-145 cells were incubated with increasing concentration of the conjugated tumor homing peptide, YRCTLNSPFFWEDMTHECHAGG-D(KLAKKLAK)₂ or control peptide D(KLAKKLAK)₂. Apoptosis was measured by staining with anti-annexin V-FITC antibody and analyzed by fluorescence microscopy.



In summary, we were successful in addressing all the questions for **Task 1**. The cumulative data generated from **Task 1** firmly support the continue characterization of the tumor-homing peptide, YRCTLNSPFFWEDMTHECHA.

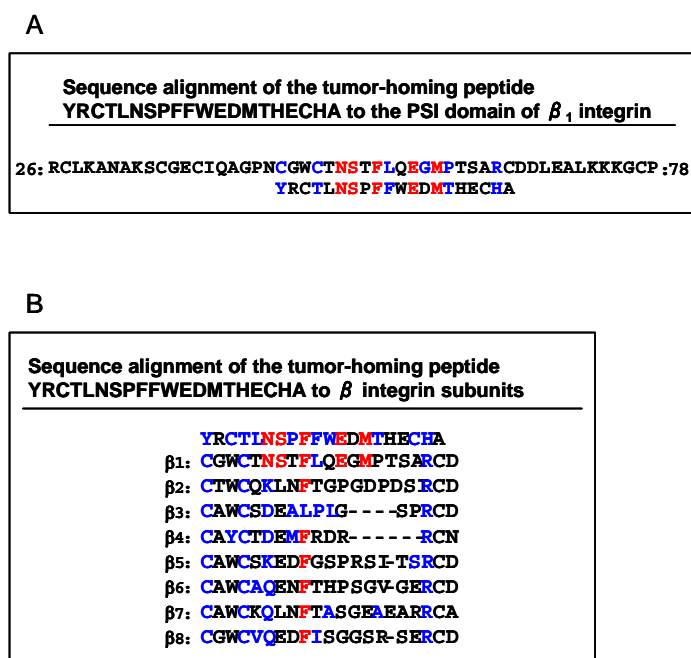
Accomplishments for task 2:

Encouraged by the data from the in vivo targeting studies and the completion of **Task 1**, we proceeded to interrogate the recovered peptides from the screening by applying bioinformatics. To determine whether the peptide sequence mimics a native protein, we performed a similarity search of the tumor-homing peptide and of the other selected peptide sequences using standard blast search engine against on-line databases followed by protein sequence alignment. Interestingly, we found that all unique phage-displayed peptides were matched to sequences present on β_1 integrin (Table 2). Unexpectedly, the dominant peptide sequence, YRCTLNSPFFWEDMTHECHA, had similarity to the plexin-semaphorin-integrin (PSI) extracellular domain (residues 26-78) of the β_1 integrin chain; moreover, we found that other selected peptides also appeared within the same region (Figure 8A). Similarities between residues are depicted in blue and 100% identity is depicted in red. The tumor-homing peptide, YRCTLNSPFFWEDMTHECHA, is highlighted in yellow. We then asked whether the similarity of the selected peptide sequence, YRCTLNSPFFWEDMTHECHA, was specific for the PSI domain of the β_1 integrin sequence or common to other known integrin β subunits. After fit analysis, and molecular modeling, we concluded that the sequence identity between YRCTLNSPFFWEDMTHECHA and the PSI domain of β_1 integrin indicated the best alignment (Figure 8B). The bioinformatic analysis supports that the tumor-homing peptide mimics the β_1 integrin PSI domain.

Table 2. Sequence alignment of tumor-homing peptides and β_1 integrin.

| Matched sequence alignment of tumor-homing peptides to β_1 integrin | |
|---|---|
| 1 | MNLQPIFWIGLIS/CCVFAQTDENRCLKNAKSCCECIQAGPNCGWCTNSTFLQIEMPTSA RCDDLEALKKKKGCPDDIENPRGSKDIKRNKRSK HTCWRDVAQPSGTVRCLK KLCYRSS TSCVRTGHDENLKAYCSS VACDISAVEELPASARSCKT GPCAATGVNPGDHGAAVCDQ LGCNKGRIWLSRLSVSCAL YRCTLNSPFFWEDMTECHA |
| 101 | GTAKLKLPEDITQIQPQQLVLRSLRSGEPQFTLKFKRAEDYPIDLYYIMDLSSYSMKDDLENVKSGLGTDLMNEMRRITSDFRIGFGSEVSKFVM SAGSELRPPEKAY VRCNE TC |
| 201 | AKLRNETSEQNCTSPFSYKVNLSLTNKGVEVFNELVGKQRIDSDSEGGEDAIMQVAVCGSLIGWRNVTRILLVFSTDAGFHFAGDGKLGIGVLPND AQLQDSGTVEHPCLR NRCMPGFLDDADSAASFCGS EVRNRALBELTNFCPY LRCPLLEVDRPNRDPAPLCSQ |
| 301 | CHLENNMYTMSHYDYDPSIAHLVQKINSEITIFAVTEEFQPVYKELKNLIPKSAVGTLSANSSNVIQLIYNLSSEVILENKLESEGVTTISKSY GNCMGLQVSELFMGPYKCRQ SRCHALRSQSVSTAGACIS ECVNSDTGVLRGAPSCLF QHCVKQFPFRESVTITCNS MH Y |
| 401 | CKNGWGTGNGRKCSNISIGDEIQFEISITSNKCPKSDSDFKIREFTFEVILQYICECECSSEGIPESEKCHENGTFECGACRCNEGRVGRHC TSQTLRGTPSLAKCSD TLCSRLEHEVGLFKPRECPF SCTRLNGTGLQNPSSACDR VCFMERQMGTDVVSFMCVN RGOWRDSTAWHVSYPVECLA WVCTSAQDTRLKEPGMCTA NCDLDDIIFLNPYTAGEFCGT ECVVSPFALSAGTSVCTI GCTEAGEVIGITTHCCGN VCCGSYCGGVEMLASRCGH GLETNNVTKVGGITRNCVG |
| 501 | ECSTDEVNSEDMYCRKENSEICSNNGECVCQCVCVRKRDNTNEIYSKFCEDNFNCDRNLICGGNGVCKCRVCECFNYTGSAOCSLDTSTCE TTCKNSMSQIMRDSRECHR IEPVINMWITPSWAHKCM NNCPEVGSQQNYSGATWCRA EGCSDIMNTAAERVTDGCSY RTQQVRSSNISPRMAIACVT TECRGASSGSVSGAATDCRD ARCREDTGFMG NDCSAHAQPGW TLCPP |
| 601 | ASNGQICNGRGICEGVCKCTDHFQGGTCEMCQTCLGVCAEHECVQCRAFNKGEKDTCTQECSEYFNITKVESRDLPQPVQDPVSHCKEKOVDCCW LGSANICTD RECGRTVHRYFWGSPESCEK BCWMASTDTGTAAHCKP DEVPMCNQ LGCMAFMLREFGATHACTQ DACSRFLGERVDTAAGCSR ASMGLGREKPRLCSDRCVLVRPEFGRGDARLCHS NQCSSLLTYQGWRKTDQIP SCQYGLIVGQPF AEHCPP CPRQFFHCTEFWPSDCSR |
| 701 | FYFTYSVNGNNEVMVMEPECTGPDILIPVAGVVAGIVLIGLALLIWKLLMIHDDRREFAKFEKEKMNKAWDNGEKS AVTTVVPKYEKG PNCYSQDGETSSHIPVQCLM IGCNHPSPLGSTVVPETCYCFK GYCTVVGAVLTIALLCVT |

Figure 8. Sequence alignment of tumor-homing peptide YRCTLNSPFFWEDMTHECHA and PSI domain. (A) The tumor-homing peptide YRCTLNSPFFWEDMTHECHA matches to the plexin-semaphorin-integrin (PSI) domain. (B) Sequence alignment of all eight β integrin-subunits and the YRCTLNSPFFWEDMTHECHA peptide sequence.



In order to further gain insight into the biological significance of the tumor-homing peptide mimicking β_1 integrin, we took a biochemical and affinity chromatography approaches to isolate and identify the corresponding binding partner. Since the tumor homing peptide, YRCTLNSPFFWEDMTHECHA, showed significant targeting to DU145 prostate tumor cells, we decided to use affinity chromatography to purify the receptor from a DU145 whole cell lysate. Our laboratory has been successful in using this approach to identify receptors (25, 26). The tumor homing peptide, YRCTLNSPFFWEDMTHECHA, and a control peptide, were immobilized on EDC/DADPA resin column. The DU145 whole cell lysate was prepared and pre-cleared on the control peptide column prior to passing the lysate onto the tumor homing peptide column. The columns were washed several times and the bound proteins were eluted and analyzed by SDS-PAGE followed by coomassie blue staining. A predominant band around 40KDa was eluted from the tumor homing peptide column (Figure 9). We excised the gel band for protein sequencing analysis by the protein core facility in MDACC. Briefly, the gel band was enzymatic ally digested and subjected to mass spectrometry followed uninterrupted fragment ion searching of non-redundant protein database. A single peptide fragment (**IFDPQNPENE**) was matched to the protein CRKL (chicken tumor virus no.10 regulator of kinase-like protein) (Figure 10).

Figure 9. Identification of receptor for the tumor homing peptide.

The tumor homing peptide, YRCTLNSPFFWEDMTHECHA, and a control peptide, CARAC, were immobilized on EDC/DADPA resin columns. The columns were washed several times prior to passing DU145 whole cell lysate through the control column prior to the tumor homing peptide column. The cell lysate was made from pooling 20 confluent plates that were lysed in 1%NP-40/PBS with protease inhibitors. The receptors bound to the peptide columns were eluted and analyzed by SDS-gel followed by coomassie blue staining.

CRKL is a 39KDa protein and contains one SH2 (Src homology domain 2) domain and two SH3 (Src homology domain 3) domains. It is involved in protein-protein interactions and signal transduction. It functions as an adaptor protein by binding to numerous signaling proteins including p130Cas, Paxillin, c-Abl, DOCK180, and C3G (27-32). This is the first report of this protein implicated in prostate cancer. Interestingly, CRKL has been shown to be involved in integrin-mediated adhesion (30).

Next, we validated the binding specificity of the tumor-homing peptide to CRKL by Western blot analysis and in vitro binding assays. We performed a Western blot analysis on the purified proteins eluted from the peptide column with the commercial available anti-CRKL antibody. We found that the eluted protein from the tumor homing peptide column was CRKL (Figure 11A). In addition, recombinant CRKL binds to the tumor homing peptide in an in vitro binding assay (Figure 11B).

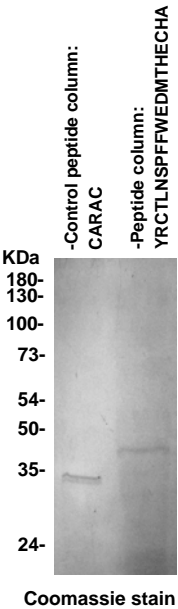
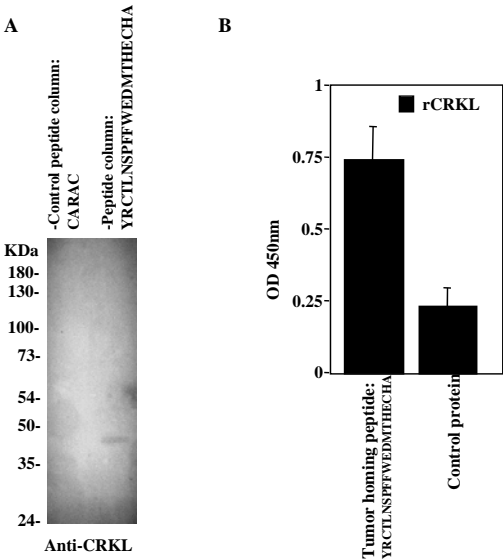


Figure 10. Protein sequence analysis of CRKL. The gel band from the tumor homing peptide column was enzymatically digested and processed for liquid chromatography electrospray ionization followed by non-redundant protein database searching. The bold region is the fragment from the mass spectrometry that matched to the protein CRKL.

CRKL (chicken tumor virus no.10 regulator of kinase-like protein)

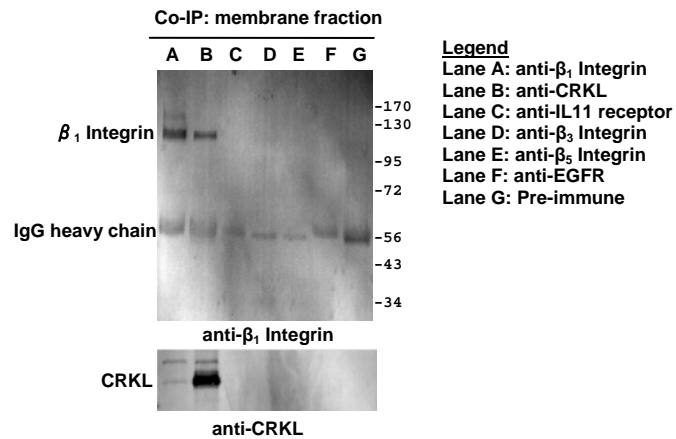
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FLVRDSSSTCPGDYVLSVS ENSRVSHYII NSLPNRR
FKIGDOEFDHLPALLEFY KIHVLDTTTLEPAPRY
PSPPMGSVSAPNLPATAEDNLEYVRTLYD PPGNDAE
DLPPFKKGEILVI IEKPPEQWWSARNKDRVGMIPV
PYVEKLVRSPPHGKHGNNRNSNSYGIPEPAHAYAQP
QTTTLPAPVSGSPGAATPLPSTQNGPVPFAKAIQK
RVPCAYDKTALALEVGDI VKVTRMNINGQWEGEVN
GRKGLFPFTHVKIFDPQNPDENE
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Figure 11. The tumor homing peptide binds to CRKL. (A) The eluted proteins from the tumor homing peptide and control columns were probed with anti-CRKL antibody by Western blotting. CRKL protein was only detected from the tumor homing peptide column. (B) The tumor homing peptide or a control protein (AHSG, alpha2-HS-glycoprotein) were immobilized on multi-well plates followed by blocking with 1% BSA and incubating with a commercial recombinant CRKL protein (Upstate Biotechnology). The complex was detected by anti-CRKL antibody (Santa Cruz Biotechnology) followed by adding TMB substrate and an ELISA machine (Bio-Tek) read the reaction at 450 nm.



Since the bioinformatics studies indicated that the tumor-homing peptide mimic the β_1 integrin, we tested this possibility by performing co-immunoprecipitation assay using DU145 cell membrane extract. We demonstrated that CRKL and β_1 integrin form a cell surface complex; in contrast, control antibodies raised against unrelated transmembrane receptors including anti-IL11 receptor, anti-EGF receptor, or other integrins (anti- β_3 , and anti- β_5) showed no association with CRKL or β_1 integrin (Figure 12).

Figure 12. Receptor validation. The association of CRKL with β_1 integrin by reciprocal co-immunoprecipitation from a membrane fraction with either anti-CRKL antibody or anti- β_1 integrin antibody. The following unrelated antibodies served as negative controls: anti-IL11R, anti-EGFR, anti- β_3 , anti- β_5 , and pre-immune serum.



In addition, we have confirmed the association between CRKL and β_1 integrin on the cell surface of the membrane by immunofluorescence and FACS analysis (Figure 13A and B). The controls showed no binding activity. The cells were not permeabilized prior to labeling in order to study cell surface localization. Since CRKL is known to be an intracellular adaptor protein, we investigated further to demonstrate that CRKL is also localized to the cell surface using well established technique-electron microscopy. We were able show that CRKL localized to the cell surface at the ultra-structural level. We found significant labeling of CRKL on the cell surface of DU145 cells by scanning and transmission electron microscopy (Figure 14A and B).

Figure 13. Cell surface localization of CRKL.

(A) Flow cytometry analysis of CRKL on DU145 cells. Immunolabeling was performed by using monoclonal anti-CRKL, anti- β_1 integrin, and anti-AHSG antibodies. (B) Immunofluorescence localization of CRKL on fixed DU145 cells by confocal microscopy. The merge image shows co-localization.

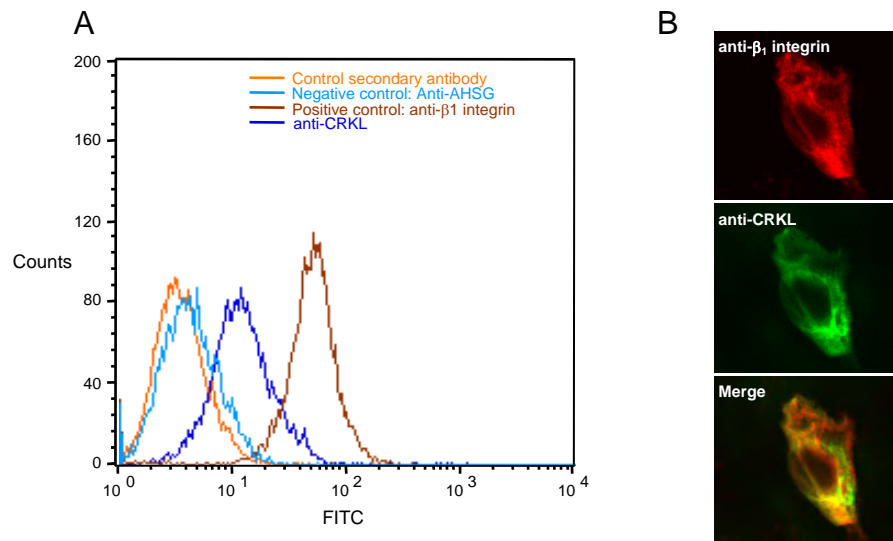
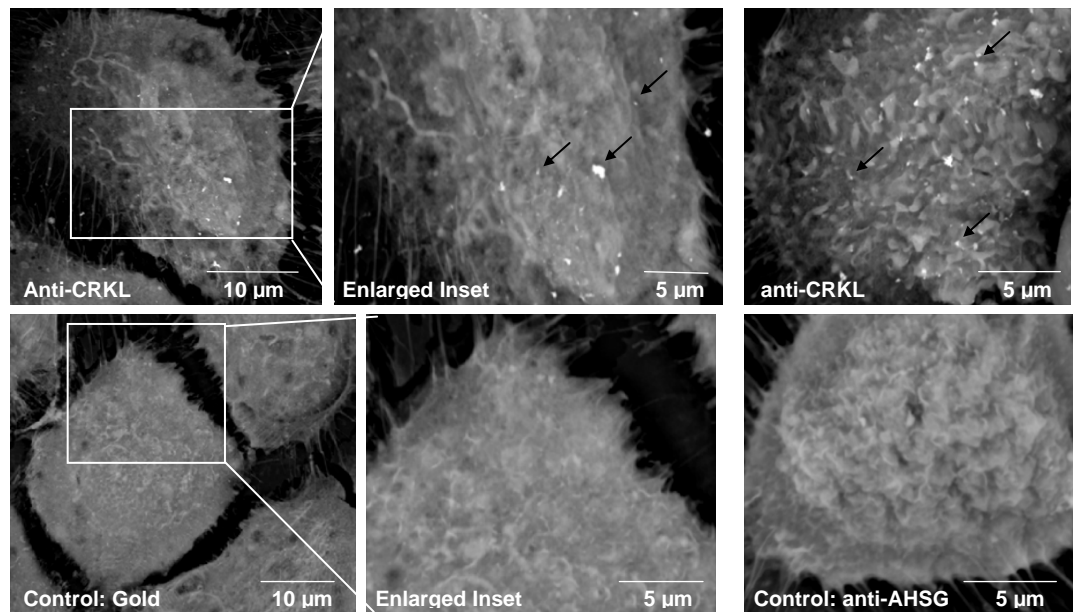
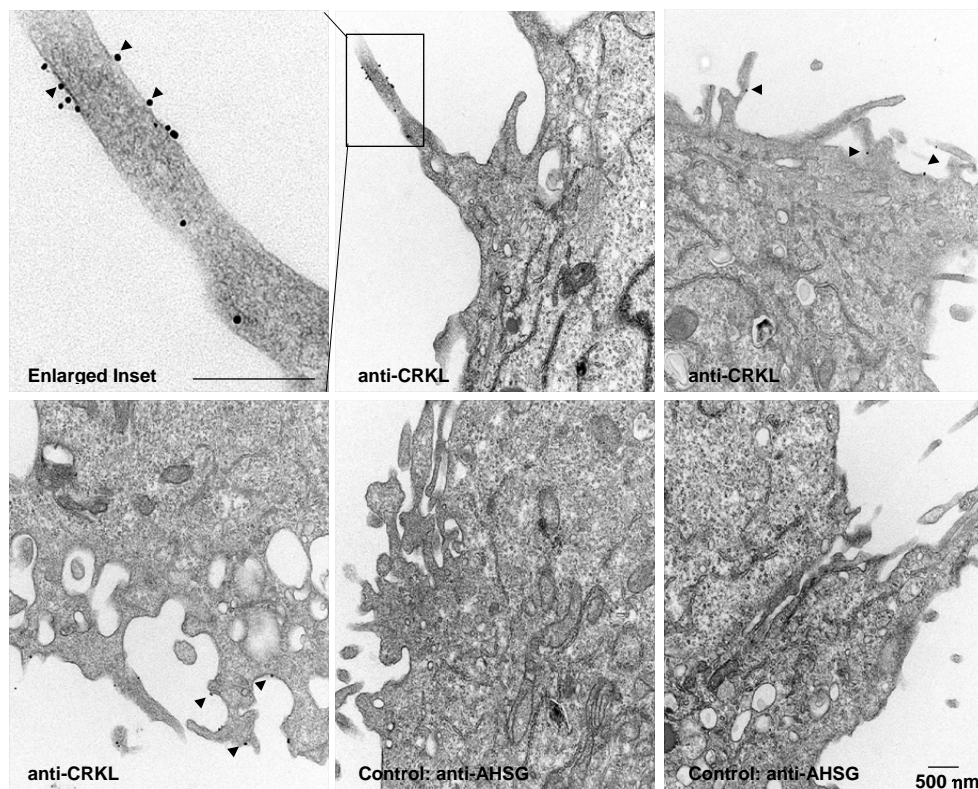


Figure 14. Cell surface localization by electron microscopy. (A) Scanning electron microscopy (SEM) of CRKL localization (arrows indicate CRKL-gold conjugated labeling). (B) Transmission electron microscopy (TEM) of CRKL showing individual CRKL-gold particles on the cell surface (arrow heads). DU145 cells were fixed without permeabilization. An anti-CRKL polyclonal and control antibody AHSG were used.

A



B



In conclusion, we have identified a new receptor-ligand complex for prostate cancer. The association between β_1 integrin and CRKL is a novel discovery and this is the first report of such finding. We plan to further functionally characterize this association in the context of the disease. If successful, the research supported by this funding mechanism may lead to new molecular targets for prostate cancer.

Materials and Methods:

Phage display random peptide library selection

In vivo phage screenings were performed as described (11, 12, 21). A random phage library displaying an insert with the general arrangement $X_2CX_{12}CX_2$ (C, cysteine; X, any residue) was systemically administered (tail vein) into athymic nude mice bearing tumor xenografts derived from human DU145 prostate cancer cells and allowed to circulate for 24 hours. With tumor-bearing mice under deep anesthesia, tumor xenografts were excised, weighed, and the bound phage population was recovered and processed as described (11, 12, 21). Three serial rounds of in vivo selection were performed.

Affinity chromatography and mass spectrometry

Standard peptide affinity columns were made by EDC and DADPA immobilization resin (Pierce). DU145 tumor cell extracts were prepared and first passed through a non-specific control peptide column followed by the tumor-homing peptide column. Columns were washed extensively, then eluted with glycine (pH 2.2), and analyzed by SDS-PAGE. Then, the gels were Coomassie-stained. A band of ~40KDa was detected and excised for protein sequencing by mass spectrometry at UTMDACC Proteomic Core Facility. The protein was identified as CRKL. Affinity purification of CRKL from serum-free condition medium was performed.

Cell surface and membrane localization

Phage cell surface binding assays were performed on DU145 cells as described, through the Biopanning and Rapid Analysis of Selective Interactive Ligands (BRASIL) methodology (22). Confocal images were acquired on an LSM 510 (Carl Zeiss) confocal microscope. DU145 cells were grown on fibronectin-coated slides, fixed with 4% paraformaldehyde (PFA) and labeled with the appropriate antibodies (polyclonal CRKL antibody and monoclonal AHSG antibody). The electron microscopy images were acquired at the High Resolution Electron Microscopy Core Facility (JSM 5900 scanning and JEM 1010 transmission electron microscopes). Gold nanoparticle antibody-conjugates (33) were prepared by mixing 20-25 nm or 40-45 nm gold in sodium borate. Gold-coupled nanoparticles were verified by TEM analysis. DU145 cells were labeled on ice with appropriate antibodies (monoclonal anti-CRKL, anti- β_1 integrin, and anti-AHSG antibodies) followed by secondary conjugated-fluorescent antibodies and analyzed by FACS at the M. D. Anderson Cancer Center (MDACC) FACS core facility.

Antibodies, peptides, cell lines, and tumor-bearing mice

All cell lines were purchased (American Tissue Type Collection; ATCC). The following antibodies were used: anti-CRKL (Santa Cruz, Cell Signaling, Epitomics or Upstate Biotechnology), anti-phospho-CRKL (Cell Signaling), anti- β_1 integrin (Chemicon or BD Transduction Laboratories), anti-IL11R (Santa Cruz), anti- β_3 and anti- β_5 integrins (25), anti-EGFR (34), anti- α_6 integrin (Chemicon), anti-AHSG/Feutrin A (R&D Systems), pre-immune serum (Jackson Laboratory). Peptides were synthesized and cyclized to our specifications (AnaSpec). Six week-old male nude mice were commercially obtained (Harlen) and tumor xenografts were generated as described (35, 36). The Institutional Animal Care and Use Committee (IACUC) at the University of Texas M. D. Anderson Cancer Center (UTMDACC) reviewed and approved all experimental procedures.

Peptide binding and internalization assays

The internalization capability of the tumor-homing peptide fused through a glycylglycine bridge to a pro-apoptotic sequence was tested as described (17, 23, 24, 35). The conjugated tumor-homing peptide, YRCTLNSPFFWEDMTHECHAGG-D(KLAKKLAK)₂ or the untargeted control peptide D(KLAKKLAK)₂ were synthesized and increasing equimolar peptide concentrations were added to the DU145 cells. Cell viability was assayed by commercially available WST-1 reagent and annexin-V staining for apoptosis (Roche) as described (24, 25). For tumor-homing phage localization studies, cells were incubated with 10^9 T.U. tumor-homing (YRCTLNSPFFWEDMTHECHA) or a negative control (Fd-tet) phage for 6 and 24 hours. Wells were washed with 20 mM glycine to remove non-specific cell surface bound phage and then fixed with 4% paraformaldehyde. The non-permeabilized cells were incubated with rabbit anti-fd bacteriophage antibody (Sigma) for 2 hours at room temperature followed by one hour incubation with Cy3-labeled anti-rabbit IgG antibody (Jackson ImmunoResearch). Cells were again fixed with 4% paraformaldehyde and mounted in the presence of DAPI (Vector Laboratories) and images were acquired with an Olympus fluorescence microscope.

Immunoprecipitation assays

Reciprocal co-immunoprecipitation from a membrane fraction was performed as described (26, 37). Immunoblots were probed with the appropriate antibodies including anti-CRKL antibody and anti- β_1 integrin antibody. The following unrelated antibodies served as negative controls: anti-IL11R, anti-EGFR, anti- β_3 , anti- β_5 , and pre-immune serum.

Sequence alignment analysis

Sequence alignment between tumor-homing phage peptides and β_1 integrin analyzed by using the Peptide Match software codified in Perl 5.8.1 based on RELIC (38). The program calculates similarity based on a predefined residue window size between an affinity selected peptide sequence and the target protein sequence from N- to C- protein terminus in one-residue shifts. The peptide-protein similarity scores for each residue were calculated based on a BLOSUM62 amino acid substitution matrix modified to adjust for rare amino acid representation. Thresholds were set at least 4 identical residues between the peptide and the protein segment to discriminate significant similarities from nonspecific background matches.

Design and construction of scrambled and mutant tumor-homing phage

To generate phage clones to study the binding properties in vivo, we designed and constructed phage displaying scrambled peptide sequences and mutants (P→A and from the selected tumor-homing phage peptide, YRCTLNSPFFWEDMTHECHA. Scrambled peptide sequences (YRFCTSPFHEWHLENTDMCA, YRECTDSPHEFHLWNTMCAF) mutants (YRCTLNSAFFWEDMTHECHA) were cloned into the SfiI-digested fUSE5 vectors (39). Briefly, 500 ng of each of the synthetic oligonucleotide templates corresponding to the displayed peptides (Sigma-Genosys) were converted to double-stranded DNA by PCR amplification with the primer set 5' GTGAGCCGGCTGCCC 3' and 5' TTCGGCCCCAGCGGC 3' (Sigma-Genosys) and 2.5 U of Taq-DNA polymerase (Promega) in 20 µl as follows: 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by 72°C for 5 min. Double-stranded DNA sequences that contained BglI restriction sites in the insert-flanking regions were purified by using a QIAquick nucleotide removal kit (Qiagen) and eluted. Oligonucleotides were digested with BglI for 2 hour at 37°C, re-purified, and ligated into SfiI-digested fUSE5 vector. The phage clones generated were PCR amplified to verify the correct insertion and nucleotide sequence. The individual phage clones were tested in phage binding assays.

In vivo tumor targeting study

In vivo targeting experiments with phage were performed as described (35, 36, 40). We used male nude mice bearing human DU145 xenografts. Briefly, mice bearing tumors (~8 mm) were anesthetized and injected intravenously via tail vein with 5×10^{10} T.U./animal of wild-type YRCTLNSPFFWEDMTHECHA-phage, or negative controls: Fd-tet phage (insertless) and scrambled YRFCTSPFHEWHLENTDMCA-phage, YRECTDSPHEFHLWNTMCAF-phage, or a mutant YRCTLNSAFFWEDMTHECHA-phage. Cohorts of two mice with size-matched tumors received each set of phage clones. After 24 hours, tumors were dissected from each mouse and phage recovered by bacterial infection and normalized by weight of tissue. The experiments were repeated twice for each tumor model.

KEY RESEARCH ACCOMPLISHMENTS

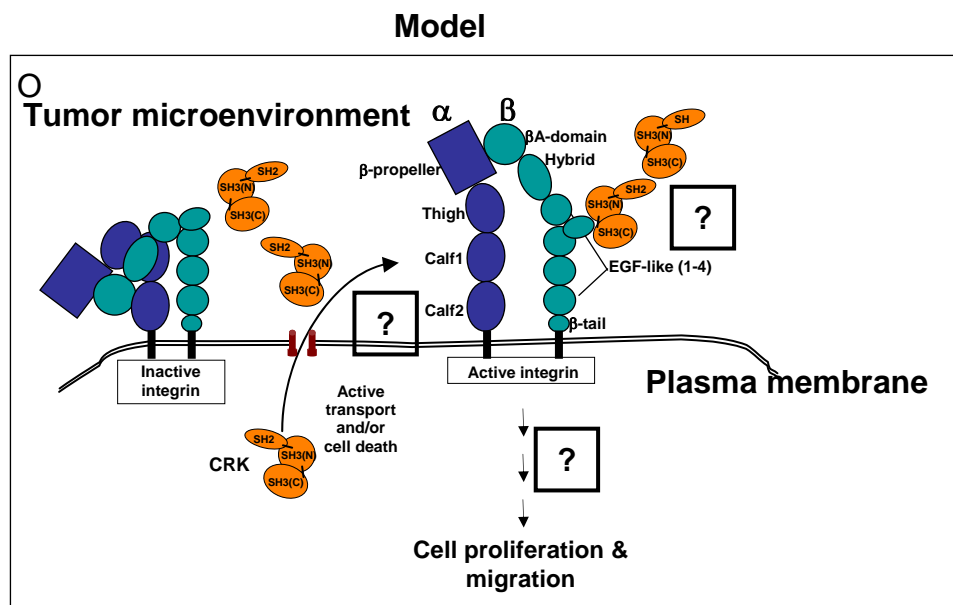
1. We have completed all the questions for Task 1.
2. We have completed most of the questions for Task 2.
3. We have identified new molecular targets for prostate cancer such as CRKL and beta1 integrin.
4. We have identified a novel complex between CRKL and beta1 integrin on the cell surface of prostate cancer cells.

REPORTABLE OUTCOMES

1. Preparation for a manuscript submission.
2. Invitation to the 11th Prouts Neck meeting on prostate cancer, 2006

CONCLUSIONS

We have identified a novel complex involving CRKL and β_1 integrin for prostate cancer. This would be another complex in addition to the androgen/androgen receptor complex for this disease. Integrins are critical receptors for cell signaling. Typically, integrin ligands include cytoskeletal and extracellular matrix (ECM) proteins. However, we found an unexpected interaction between a signaling adapter protein-CRKL and β_1 integrins on the cell surface. This is surprising because CRKL has been considered an intracellular signaling molecule while integrins are thought of as a regulatory protein involved in extracellular matrix signaling. It is likely that extracellular CRKL may have multiple functional roles as yet unrecognized in the tumor microenvironment. From our discoveries more new questions have been raised and warrant further investigation. We propose a working model to explain such a complex and a possible mechanism (figure below). One might speculate that extracellular CRKL (secreted and/or released) can perhaps function as an autocrine or paracrine factor within tumors. This in turn activates a cell signaling cascade down stream through the integrin-mediated pathway.



As with new findings, more new questions are asked such as how is the intracellular CRKL being secreted or what domains are involved in the extracellular interaction between CRKL and integrins (indicated as a question mark in the model). And most importantly, what are the signaling molecules involved downstream of the complex that are functionally significant. Through more experiments and funding we may be able to address some of these new interesting questions.

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APPENDICES

None reported.